Advantages of using Intelligent Sample Loading with the HPLC-Chip for automated sample enrichment

Application Note

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Abstract

The Agilent HPLC-Chip integrates enrichment column, analytical column, associated connection capillaries and a nanospray emitter directly on a single, small, reusable microfluidic chip and the HPLC-Chip and companion HPLC-Chip Cube interface provide an easy to use, robust and reliable nanospray LC/MS platform when compared to conventional nanocolumn LC/MS technology. When using an enrichment column, appropriate selection of valve timetable events is critical for proper loading and transfer of the sample to the analytical nanocolumn and requires re-adjustment each time the injection volume is changed. Optimization of this process is often time consuming and improper settings can significantly degrade the efficiency of the enrichment process. This Application Note will demonstrate how Intelligent Sample Loading automates and optimizes sample enrichment on the HPLC-Chip. Direct comparison with the timetable approach will also show how Intelligent Sample Loading increases the number of peptides detected in a protein digest test mixture.

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Introduction

Microfluidic HPLC-Chip/MS technology is rapidly establishing itself as a robust, reliable easy to use alternative to conventional nanocolumn LC-MS system[1]. Integration of the enrichment column, analytical columns, connecting capillaries and nanospray emitter directly on to the micro-fluidic HPLC-Chip has demonstrated improved sensitivity and enhanced chromatographic performance leading to improved peptide and protein identification[2-3]. HPLC-Chips and the HPLC-Chip Cube interface have been used with Ion-Trap and TOF mass spectrometers. Recent applications using HPLC-Chip/MS technology include biomarker discovery[4], investigation of the nucleolar proteome[5], phophoproteome analysis[6] and oligosaccharide separations in mucins and human milk[7]. When using nanocolumn LC/MS, long injection times and very small or very dilute samples requires the use of an enrichment column. This enrichment column is used to concentrate the sample and also allows loading the sample at higher flow rates reducing the overall injection cycle time. In addition, biological samples can be desalted on the enrichment column to minimize the introduction of salt in the MS source. Flow switching is required for sample loading on to the enrichment column and subsequent transfer to the analytical nanocolumn. With the HPLC-Chip/MS system, this is achieved using a novel microvalve system contained in the HPLC-Chip Cube interface that ensures seamless zero dead volume leak tight connections with the HPLC-Chip. Automation and optimization of the flow switching process is critical for proper loading of the sample on to the enrichment column and transfer to the analytical nanocolumn. For conventional nanocolumn LC/MS system, valve control is typically performed using a valve event timetable. However, this approach can create unnecessarily large data files and requires re-optimization of the valve event timetable each time the sample injection volume is modified. This process is quite often time consuming and error prone and improper settings can significantly degrade the efficiency of the enrichment process. This will negatively impact both the amount and number of compounds retained on the enrichment column. In this Application Note, the features and performance of Intelligent Sample Loading will be discussed in detail. Direct comparison with the time table approach will show how Intelligent Sample Loading increases the number of peptides detected in a protein digest test mixture and reduces data file size.

Equipment, sample and data processing

The Agilent 1200 HPLC-Chip/MS system includes the following components:

- Agilent 1200 Series nanoflow LC system consisting of the nanoflow pump, micro vacuum degasser, and thermostatted micro well-plate autosampler (µ-WPS)
- Agilent 1200 Series capillary pump for sample loading and desalting
- Agilent 1200 HPLC-Chip Cube MS interface
- Agilent LC/MSD Trap XCT Ultra mass spectrometers
- Agilent Protein ID chip
- Spectrum Mill Protein ID sofware

Chromatographic conditions:

HPLC-Chip/MS:	
Enrichment column:	ZORBAX 300SB-C18,40 nL, 5 µm
Analytical column:	ZORBAX 300SB-C18, 75 µm x 43 mm, 5 µm
Loading flow rate:	4 µL/min
Loading mobile phase:	3 % acetonitrile, 0.2 % formic acid
Injection flush volume:	6 µL for IFV method and 0 µL for TT method respectively
Injection volume:	5 µL
Valve positions (for TT method):	0 min enrichment, 6 min analysis, 69 min enrichment
Flow rate:	300 nL/min
Mobile phase:	A = 0.2 % formic acid in water
	B = 0.2 % formic acid in acetonitrile

Gradient:	TT method:		IFV method:	
	Time (min)	% B	Time (min)	% B
	0	8	0	8
	6	8	57	40
	63	40	61	60
	67	60	62	60
	68	60	63	8
	69	8	Stop time: 64	
	Stoptime: 75		Post time: 10 min	
	Post time: 5 min			

Needle flush solvent: 20 % methanol + 0.2 % formic acid in water

Table 1
HPLC-Chip chromatographic conditions.

For peptide detection and clustering, a script was developed for conversion of the Agilent Ion-Trap data files into text files containing information on all m/z, intensity and retention time values above a user specified threshold. The resulting text file is then processed using proprietary software that enables data reduction, peptide detection and alignment of peptide ion maps (m/z, retention time, intensity)[8]. Protein and peptide identification was performed using Spectrum Mill. The sample used for this evaluation was a peptide mixture from the tryptic digest of 8 different proteins (bovine serum albumin, rabbit aldolase, yeast alcohol dehydrogenase, bovine catalase, bovine glutamate dehydrogenase, E.Coli glycerokinase, human lactotransferrin, bovine lactoperoxidase, Michrom Bioresources, Auburn, CA). The digests were dissolved in water with 5 % acetonitrile and 0.2 % formic acid, and mixed together to reach the concentration of 40 fmol/µL each. The final injection volume contained 80 ng of the mixture (ProMix). Chromatographic and Ion Trap MS conditions for the experiments are described in tables 1 and 2.

Chromatographic conditions:	
Ionization mode:	HPLC-Chip/MS interface
Drying gas flow:	3.5 L/min
Drying gas temperature:	300 °C
Capillary voltage:	1900 V
Skimmer 1:	30 V
Capillary exit:	75 V
Trap drive:	85
Averages:	1
ICC: On	
Maximum accumulation time:	150 ms
Smart target:	500000
MS scan range:	400-1600
Automatic MS/MS:	Peptide scan mode (standard-enhanced for MS and ultra-scan for MS/MS)
Number of precursors:	3
Averages:	1
Fragmentation amplitude:	1.3 V
Active exclusion:	On, 2 spectra, 1 min
Prefer +2:	On
MS/MS scan range:	100-2200
ICC target:	500000

Table 2
Ion Trap MS conditions.

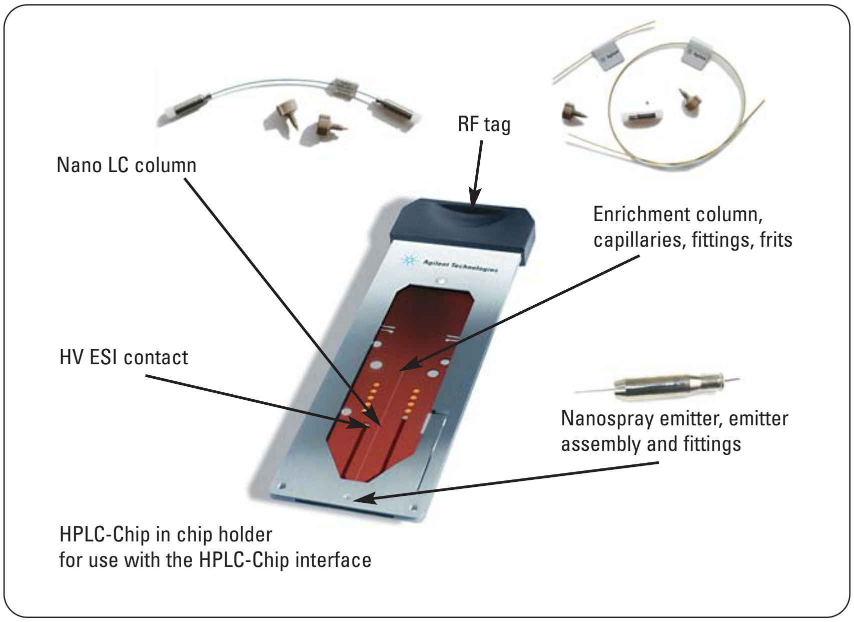

Figure 1
Design of the Protein ID chip. The different elements necessary for nanoflow LC/MS are integrated on the HPLC-Chip.

Workflow and description of the HPLC-Chip

The chip fabrication process and the chip interface for MS have been previously described[3,9] and for this work, the Protein ID (p/n G4240-62001) chip was used. This chip includes a 40-nL enrichment column and a 75-µm x 43-mm analytical column which are both packed with 5-µm 300A ZORBAX SB-C18. Figure 1 shows the components integrated onto this microfluidic HPLC-Chip. Components and workflows integrated on to the HPLC-Chip are outlined in figure 2 and clearly illustrate the requirement for flow switching so that the sample can

be loaded using higher flow rates onto the enrichment column, flushed onto the analytical column, and then analyzed by nanoflow LC/MS.

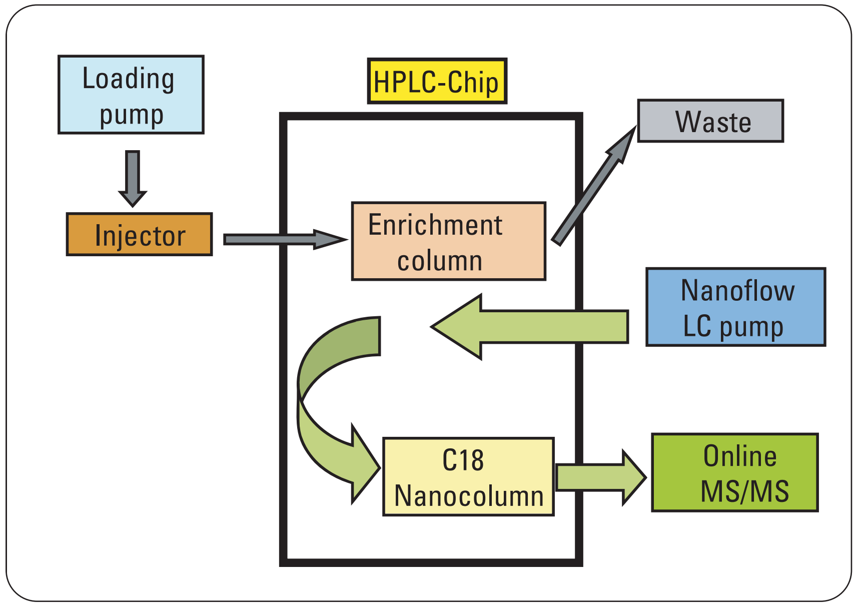

Figure 2
Workflow diagram for the HPLC-Chip/MS system.

Intelligent sample loading

The HPLC-Chip/MS system included a novel Intelligent Sample Loading (ISL) feature that decouples the sample loading process from the LC/MS analysis. ISL will allow the system to automatically calculate the required time to effectively load the sample. At the end of this time, the HPLC-Chip Cube microvalve will automatically switch from enrichment to analysis mode and the pump gradient and MS data acquisition will begin. This novel feature eliminates the need to adjust the method when changing the injection volume and saves time by using the shortest possible injection cycle. Furthermore, data file size is also reduced since the MS data acquisition coincides with the switching from enrichment to analysis mode instead of starting at the beginning of the autosampler sample loading cycle.

Intelligent Sample Loading parameters

Automatic calculation of the optimum sample loading time requires that the HPLC-Chip/MS system has the ability to measure actual flow rates from the sample loading pump and prior knowledge of the capillary tubing delay volume between the autosampler seat and the enrichment column on the HPLC-Chip. This delay volume plus a flush factor are defined as the injection flush volume (IFV) and is calculated as follows:

IFV = *(VSeatCap +VTransferCap) x factor*

where:

VSeatCap = delay volume of the µ-WPS seat capillary
VTransferCap = delay volume of the capillary from µ-WPS to Chip Cube
factor: number of time to flush the delay volume.

Typical values are:

VSeatCap: 75 µm x 150 mm (blue) = 0.663 µL
VSeatCap: 100 µm x 150 mm (black) = 1.178 µL
VTransferCap: 25 µm x 1050 mm (yellow) = 0.515 µL
factor = 3 (typical flush factors are in the range of 2 to 6)

For this experiment, the HPLC-Chip Cube was used with 100-µm seat capillary and the IFV is: (1.178 µL + 0.515 µL) x 3,5 = 5.926 µL.

Measurement of the actual flow rate from the capillary loading pump is achieved by linking the loading pump to the HPLC-Chip Cube during the initial system setup. Linkage of the capillary loading pump to the Chip Cube will allow the Chip Cube to monitor real time flow from the capillary loading pump and automatically adjust the sample loading time when the injection volume is changed. Sample loading time is automatically calculated by the system as follows:

Sample loading time = IFV + injection volume / Actual capillary loading pump flow

Thus, given an IFV of 5.926 µL, an injection volume of 1 µL and a flow rate of 4 µL/min at the capillary loading pump:

Sample loading time:
(5.926 µL + 1 µL) / 4 µL/min = 1.73 min

Figure 3 illustrates the dialog box from the Chemstation pump configuration pull-down menu required to link the capillary loading pump and the nanoflow pump to the HPLC-Chip Cube. In addition to allowing real time measurement of flows, linking both pumps provide an additional safety function by allowing them to react to the status of the Chip Cube and turn the flow off when the chip is not in Operate (spraying) position. This ensures that no solvent is spilled into the Chip Cube while the chip tip is retracted or when the chip is unloaded and the valve stator ports are open.

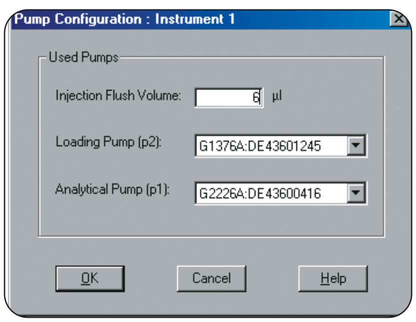

Figure 3
Pump configuration dialog box.

Changing the injection volume

A change to the injection volume specified in the method or autosampler sequence table will automatically trigger a recalculation of the sample loading time. No other operator input is required. Over time, the enrichment column flow characteristics will change and the ability to perform real time measurement of the capillary loading pump flow ensures continued optimized sample loading time throughout the lifetime of the HPLC-Chip. This ensures that changing backpressures (and flows) will not affect the sample transfer.

Smaller data file size

Decoupling the sample loading process from the LC/MS data acquisition reduces the data file size since data acquisition is started at the same time the sample is transferred to the analytical nanocolumn instead of at the beginning of the sample injection cycle. Figure 4 highlights the difference in run time achieved when using ISL compared to a microvalve time table method for the ProMix tryptic peptide sample. In this example, data acquisition time could be reduced by 16 % (from 51 to 43 min) and data file size by 11 %. Reducing the acquisition time will increase throughput. Smaller data files will accelerate data processing time and save valuable disk space on the host computer system.

Identify more peptides with ISL

Flow switching timing is critical for optimum loading of the sample on to the enrichment column and transfer to the analytical nanocolumn. Unfortunately, the process of selecting flow switching parameters can be time consuming and require multiple experiments to select and optimize all parameters. Because of this, sample loading times are often set arbitrarily following a limited set of optimization experiments. Improper settings of the sample loading time will significantly degrade the efficiency of the enrichment process and negatively impact both the amount and number of compounds retained on the enrichment column.

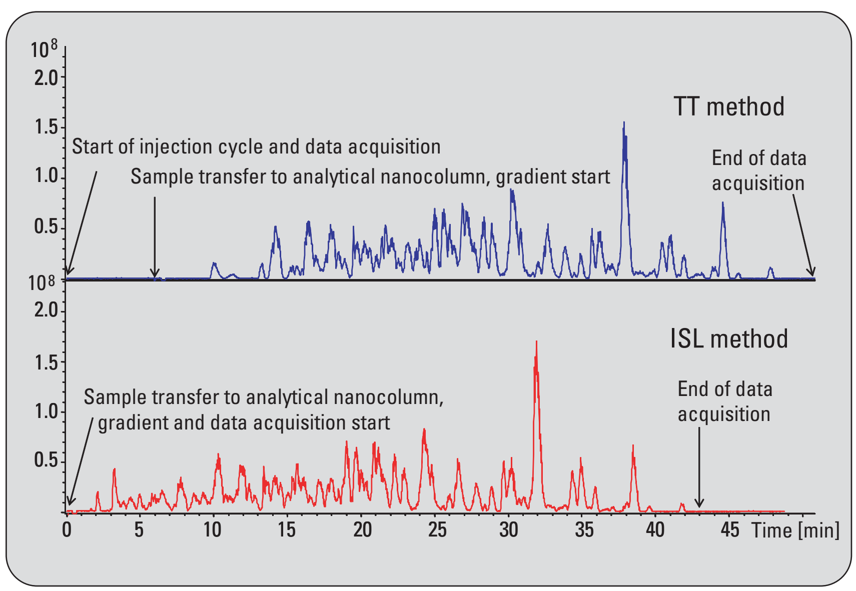

Figure 4
ProMix tryptic digest peptide sample using TimeTable (upper, TT) and ISL (lower, ISL). Sample analysis time was decreased by 18 %. Data file size was reduced by 11 %.

Figures 5 and 6 clearly demonstrate this problem. In figure 5, the base peak chromatogram (BPC) of ProMix is compared using both a manual timetable (TT) and ISL approach. For the most part, the BPC's of both the TT and ISL runs appear to be identical. However, significant differences are noticeable in the initial part of the BPC with several more peaks observed in the sample analysed using ISL (figure 5). This is further highlighted with the comparison of the peptide ion maps generated from both the TT and ISL runs (figure 6). Zooming in on the initial segment of the peptide ion map of each run, we can clearly observe the additional peptides detected when using the ISL approach. For this example with the 8 protein ProMix tryptic digest sample, we were able to detect 6.1 % more peptides using ISL than with the TT approach. Table 3 highlights a list of additional peptides detected. Because of the overall chromatographic performance observed for the TT approach (figure 6, left), the fact that the sample loading process was not fully optimized could have passed unnoticed and resulted in significant loss of information from the sample. This demonstrates the

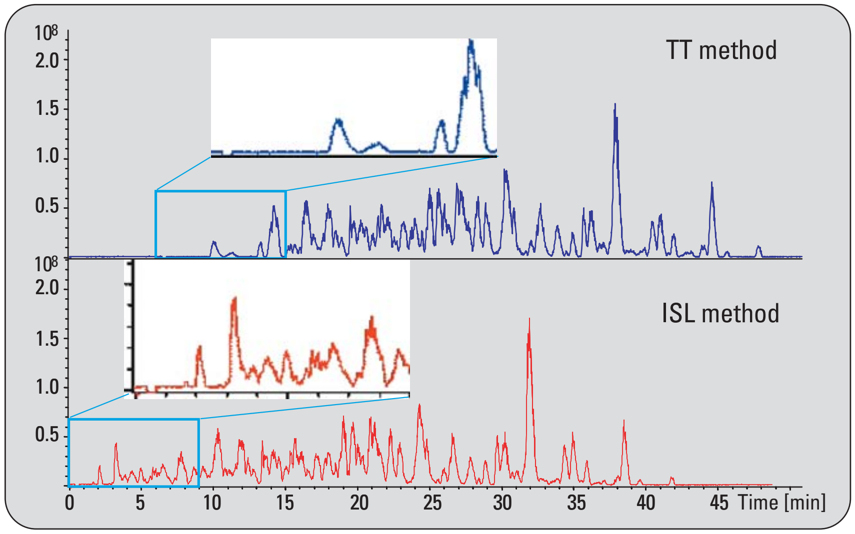

Figure 5
BPC of ProMix with TT (top) and ISL (bottom) with scale expansion for first part of the BPC. Additional peaks are clearly visible in the ISL chromatogram.

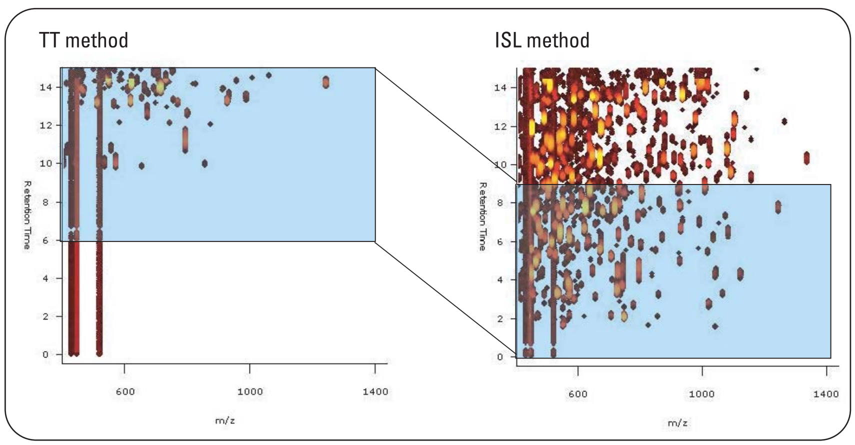

Figure 6
Peptide ion map of ProMix with TT (left) and ISL (right). 6.1 % more peptides were detected using ISL. Sample loading on to the analytical nanocolumn occurred at t = 6 min for the TT approach and t = 0 for the ISL.

	m/z measured (Da)	MH^+ Matched (Da)	MH^+ Error (Da)	Sequence	RT (min)	Intensity	Protein
1	667.83	1334.67	0.02	R.EFRPGIETTER.N	6.19	6.37e+008	Glycerol kinase
2	503.34	1004.67	0.15	R.ETTIVWEK.E	9.00	0.90e+007	Glycerol kinase
3	625.35	1249.62	0.09	R.FKDLGEEHFK.G	6.67	3.01e+008	BSA
4	722.94	1443.87	0.25	K.YICDNQDTISSK.L	4.4	0.83e+007	BSA
5	745.90	1490.71	0.08	R.LQSIGTENTEENR.R	2.65	2.51e+007	Aldolase
6	481.82	962.57	0.07	K.DAQLFZIKK.A	8.94	7.56e+007	Catalase
7	656.43	1310.85	0.17	K.RLCENIAGHLK.D	9.2	1.2e+007	Catalase
8	740.36	1479.68	0.03	R.FNSANDDNVTQV.R	4.58	2.03e+008	Catalase
9	575.90	1149.79	0.17	R.THYYAVAVVK.K	8	1.1e+007	Lactotransferrin
10	498.43	994.85	0.32	R.RFTMELAK.K	9.1	0.70e+007	Glutamate dehydrogenase
11	522.86	1043.71	0.14	R.QLLLTADDR.V	9	3.90e+007	Aldolase

Table 3
Additional ProMix peptides detected using Intelligent Sample Loading.

critical aspect of optimizing sample loading times and the advantages of using the automated ISL feature of the HPLC-Chip/MS system.

Conclusions

Intelligent Sample Loading optimizes sample loading on to the enrichment column and transfer to the analytical nanocolumn when using the HPLC-Chip. Automation of the entire process ensures that the operator can change injection volumes without the need to re-optimize method parameters for sample loading. By automatically selecting the best parameters, ISL shortens the analysis time and reduces the data file size, saving disk storage space and accelerating the data processing of the sample. Compared to a time table method, ISL automatically selects the most efficient sample loading parameters allowing for the detection of additional peptides missed when using the timetable method.

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